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CLAIM FOR PRIORITY UNDER 35 U.S.C. § 119

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19,498 — PTO Reg. No.

Signature
May 9, 2001 — Date of Signature

Assistant Commissioner for Patents

Washington, D.C. 20231

Sir:

A claim for priority is hereby made under the provisions of 35 U.S.C. § 119 for the above-identified PCT application corresponding to China Application CN 98111041.X filed August 31, 1998 based upon International Application PCT/CN99/00132 filed August 30, 1999.

Respectfully submitted,

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A NOVEL HUMAN LYSOZYME GENE, ITS ENCODED POLYPEPTIDE AND THE METHOD FOR PREPARING THEM

The invention relates to a new polynucleotide, the polypeptide encoded by said polynucleotide, the uses of said polynucleotide and polypeptide, and the methods for preparing same. In particular, the polypeptide of the invention is identified as a new member of the lysozyme family.

Lysozyme exists ubiquitously in all parts of organisms, including various tissues, organs, and sera; it is especially abundant in egg white. Lysozyme is mainly secreted by the epithelial cell of certain glands and some kinds of leukocyte.

Lysozyme was first reported by Fleming, et al. in 1922. Afterward, lysozyme has been widely studied. A lot of papers concerning its crystal structure, protein catalytic domains, catalytic dynamics, immunology, molecular evolutionary, and so on, have been published. Lysozyme is one of the proteins that are studied most extensively and intensively. However, the study on lysozyme gene is not yet sufficient. Nowadays, only a few lysozyme genes from different species, such as E.coli T4, salmonella P22 phage, bacillus Φ phage and chicken, etc., have been cloned. (1983 J. Mol. Biol. 165. 229-248; 1985 Virology 143, 280-289; 1987 Proc. Natl. Acad. Sci. USA, 77, 5759-5763). The cloning about human lysozyme gene was also reported (1988, Gene 66,223-234).

The main function of lysozyme is to hydrolyze the beta(1-4) glycosidic bond between N-acetylmuramic acid (NAM) and N-acetylgluconic acid (NAG) of the bacterial cell wall. In the organism, lysozyme can act as a nonspecific immune molecule against bacterial infections, and as a digestive enzyme in enteron and some mollusks which live on bacteria. Further, lysozyme has the function of inhibiting tumor growth. Therefore, lysozyme has important applications in both industry and medicine.

One purpose of the invention is to provide a new polynucleotide which encodes a new member of lysozyme gene family. The new human lysozyme is named LYC3.

Another purpose of the invention is to provide a new member of lysozyme protein family, which is named LYC3.

Still another purpose of the invention is to provide a new method for preparing said new human lysozyme by recombinant techniques.

The invention also relates to the uses of said human lysozyme and its coding sequence.

In one aspect, the invention provides an isolated DNA molecule, which comprises a nucleotide sequence encoding a polypeptide having human LYC3 protein activity, wherein said nucleotide sequence shares at least 70% homology to the nucleotide sequence of nucleotides 81-521 in SEQ ID NO: 3, or said nucleotide sequence can hybridize to the nucleotide sequence of nucleotides 81-521 in SEQ ID NO: 3 under moderate stringency. Preferably, said nucleotide sequence encodes a polypeptide comprising the amino acid sequence of SEQ ID NO: 4 , and more preferably, the sequence comprisessequencein SEQ ID NO: 4.

Further, the invention provides an isolated LYC3 polypeptide, which comprises a polypeptide having the amino acid sequence of SEQ ID NO: 4 , its active fragments, and its active derivatives. Preferably, the polypeptide is a polypeptide having the amino acid sequence of SEQ ID NO: 4 .

The invention also provides a vector comprising said isolated DNA.

The invention further provides a host cell transformed with said vector.

In another aspect, the invention provides a method for producing a polypeptide with the activity of LYC3 protein, which comprises:

(a) forming an expression vector of LYC3 protein comprising the nucleotide sequence encoding the polypeptide having the activity of LYC3 protein, wherein said nucleotide sequence is operably linked with an expression regulatory sequences, and said nucleotide sequence shares at least 70% homology to the nucleotide sequence of positions 81-521 in SEQ ID NO: 3;

(b) introducing the vector of step (a) into a host cell, thereby forming a recombinant cell of LYC3 protein;

(c) culturing the recombinant cell of step (b) under the conditions suitable for the expression of LYC3 polypeptides;

(d) isolating the polypeptides having the activity of LYC3 protein.

In one embodiment of the present invention, the isolated polynucleotide has a full length of 544 nucleotides, whose detailed sequence is shown in SEQ ID NO: 3. The open reading frame (ORF) locates at nucleotides 81-521.

In the present invention, the term "isolated" or "purified" or "substantially pure" DNA refers to a DNA or fragment which has been isolated from the sequences which frank it in a naturally occurring state. The term also applied to DNA or DNA fragment which has been isolated from other components naturally accompanying the nucleic acid and from proteins naturally accompanying it in the cell.

In the present invention, the term "LYC3 protein encoding sequence" or " LYC3 polypeptide encoding sequence " refers to a nucleotide sequence encoding a polypeptide having the activity of LYC3 protein, such as the nucleotide sequence of positions 81-521 in SEQ ID NO: 3 or its degenerate sequence. The degenerate sequences refer to the sequences formed by replacing one or more codons in the ORF of 81-521 in SEQ ID NO: 3 with degenerate codes which encode the same amino acid. Because of the degeneracy of codon, the sequence having a homology as low as about 70% to the sequence of nucleotides 81-521 in SEQ ID NO: 3 can also encode the sequence shown in SEQ ID NO: 4. The term also refers to the nucleotide sequences that hybridize with the nucleotide sequence of nucleotides 81-521 in SEQ ID NO: 3 under moderate stringency or preferably under high stringency. In addition, the term also refers to the sequences having a homology at least 70%, preferably 80%, more preferably 90% to the nucleotide sequence of nucleotides 81-521 in SEQ ID NO: 3.

The term also refers to variants of the sequence in SEQ ID NO: 3, which are capable of coding for a protein having the same function as human LYC3 protein. These variants includes, but are not limited to: deletions, insertions and/or substitutions of several nucleotides (typically 1-90, preferably 1-60, more preferably 1-20, and most preferably 1-10) and additions of several nucleotides (typically less than 60,

preferably 30, more preferably 10, most preferably 5) at 5' end and/or 3' end.

In the present invention, "substantially pure" proteins or polypeptides refers to those which occupy at least 20%, preferably at least 50%, more preferably at least 80%, most preferably at least 90% of the total sample material (by wet weight or dry weight). Purity can be measured by any appropriate method, e.g., in the case of polypeptides by column chromatography, PAGE or HPLC analysis. A substantially purified polypeptides is essentially free of naturally associated components.

In the present invention, the term "LYC3 polypeptide "or "LYC3 protein " refers to a polypeptide having the activity of LYC3 protein comprising the amino acid sequence of SEQ ID NO: 4 . The term also comprises the variants of said amino acid sequence which have the same function of human lysozyme. These variants include, but are not limited to, deletions, insertions and/or substitutions of several amino acids (typically 1-50, preferably 1-30, more preferably 1-20, most preferably 1-10), and addition of one or more amino acids (typically less than 20, preferably less than 10, more preferably less than 5) at C-terminal and/or N-terminal. For example, the protein function are usually unchanged when an amino residue is substituted by a similar or analogous one. Further, the addition of one or several amino acids at C-terminal and/or N-terminal will not change the function of protein. The term also includes the active fragments and derivatives of LYC3 protein.

The variants of polypeptide include homologous sequences, allelic variants, natural mutants, induced mutants, proteins encoded by DNA which hybridizes to LYC3 DNA under high or low stringency conditions as well as the polypeptides or proteins retrieved by antisera raised against LYC3 polypeptide. The present invention also provides other polypeptides, e.g., fusion proteins, which include the LYC3 polypeptide or fragments thereof. In addition to substantially full-length polypeptide, the soluble fragments of LYC3 polypeptide are also provided. Generally, these fragments comprise at least 10, typically at least 30, preferably at least 50, more preferably at least 80, most preferably at least 100 consecutive amino acids of human LYC3 polypeptide.

The present invention also provides the analogues of LYC3 protein or polypeptide. Analogues can differ from naturally occurring LYC3 polypeptide by amino acid sequence differences or by modifications which do not affect the sequence, or by both. These polypeptides include genetic variants, both natural and induced. Induced variants can be made by various techniques, e.g., by random mutagenesis using irradiation or exposure to mutagens, or by site-directed mutagenesis or other known molecular biologic techniques. Also included are analogues which include residues other than those naturally occurring L-amino acids (e.g., D-amino acids) or non-naturally occurring or synthetic amino acids (e.g., beta- or gamma-amino acids). It is understood that the polypeptides of the invention are not limited to the representative polypeptides listed hereinabove.

Modifications (which do not normally alter primary sequence) include in vivo, or in vitro chemical derivation of polypeptides, e.g., acelylation, or carboxylation. Also included are modifications of glycosylation, e.g., those made by modifying the glycosylation patterns of a polypeptide during its synthesis and processing or in the further processing steps, e.g., by exposing the polypeptide to enzymes which affect glycosylation (e.g., mammalian glycosylating or deglycosylating enzymes). Also included are sequences which have phosphorylated amino acid residues, e.g., phosphotyrosine, phosphoserine,

phosphothronine, as well as sequences which have been modified to improve their resistance to proteolytic degradation or to optimize solubility properties.

The invention also includes antisense sequence of the sequence encoding LYC3 polypeptide. Said antisense sequence can be used to inhibit expression of LYC3 in cells.

The invention also include probes, typically having 8-100, preferably 15-50 consecutive nucleotides. These probes can be used to detect the presence of nucleic acid molecules coding for LYC3 in samples.

The present invention also includes methods for detecting LYC3 nucleotide sequences, which comprises hybridizing said probes to samples, and detecting the binding of the probes. Preferably, the samples are products of PCR amplification. The primers in PCR amplification correspond to coding sequence of LYC3 polypeptide and are located at both ends or in the middle of the coding sequence. In general, the length of the primers is 20 to 50 nucleotides.

A variety of vectors known in the art, such as those commercially available, are useful in the invention.

In the invention, the term "host cells" includes prokaryotic and eukaryotic cells. The common prokaryotic host cells include Escherichi coli, Bacillus subtilis, and so on. The common eukaryotic host cells include yeast cells, insect cells, and mammalian cells. Preferably, the host cells are eukaryotic cells, e.g., CHO cells, COS cells, and the like.

In another aspect, the invention also includes antibodies, preferably monoclonal antibodies, which are specific for polypeptides encoded by LYC3 DNA or fragments thereof. By "specificity" is meant an antibody which binds to the LYC3 gene products or a fragments thereof. Preferably, the antibody binds to the LYC3 gene products or a fragments thereof and does not substantially recognize and bind to other antigenically unrelated molecules. Antibodies which bind to LYC3 and block LYC3 protein and those which do not affect the LYC3 function are included in the invention. The invention also includes antibodies which bind to the LYC3 gene product in its unmodified as well as modified form.

The present invention includes not only intact monoclonal or polyclonal antibodies, but also immunologically-active antibody fragments, e.g., a Fab' or $(Fab)_2$ fragment, an antibody light chain, an antibody heavy chain, a genetically engineered single chain Fv molecule (Lander, et al.,US Pat No. 4,946,778), or a chimeric antibody, e.g., an antibody which contains the binding specificity of a murine antibody, but the remaining portion of which is of human origin.

The antibodies in the present invention can be prepared by various techniques known to those skilled in the art. For example, purified LYC3 gene products, or its antigenic fragments can be administrated to animals to induce the production of polyclonal antibodies. Similarly, cells expressing LYC3 or its antigenic fragments can be used to immunize animals to produce antibodies. Antibodies of the invention can be monoclonal antibodies which can be prepared by using hybridoma technique (See Kohler, et al., Nature, 256; 495,1975; Kohler, et al., Eur. J. Immunol. 6: 511,1976; Kohler, et al., Eur. J. Immunol. 6: 292, 1976; Hammerling, et al., In Monoclonal Antibodies and T Cell Hybridomas, Elsevier, N.Y., 1981). Antibodies of the invention comprise those which block LYC3 function and those which do not affect LYC3 function. Antibodies in the invention can be produced by routine immunology techniques and using fragments or functional regions of LYC3 gene product. These fragments and functional regions can be

prepared by recombinant methods or synthesized by a polypeptide synthesizer. Antibodies binding to unmodified LYC3 gene product can be produced by immunizing animals with gene products produced by prokaryotic cells (e.g., E. coli); antibodies binding to post-translationally modified forms thereof can be acquired by immunizing animals with gene products produced by eukaryotic cells (e.g., yeast or insect cells).

In one embodiment, the polynucleotide of the invention is 544 bp in full length whose detailed sequence is shown in SEQ ID NO: 3 with the ORF located at positions 81-521. Said polynucleotide was obtained as follows: human brain λ gt 11 cDNA library (Clontech) was used as a template and PCR was carried out with the synthetic forward primer A1 : 5'-AGAGTGGTGGTGGCTCCACTCTG-3' and reverse primer B :5'-TGCTGTGCATGGTTCCGTCCATC-3'. A target fragment of 544bp was obtained. The sequencing of the PCR product gave the full length cDNA sequence shown in SEQ ID NO: 3.

Homology comparison showed that the nucleotide sequence and the coded protein sequence of the invention shared remarkable homology to other lysozymes from different origins. Therefore, it indicates it is a new member of lysozyme family and has some important functions of the family.

Lysozyme can lyse cells by hydrolyze the beta(1-4) glycosidic bond between N-acetylmuramic acid (NAM) and N-acetylgluconic acid (NAG) of the bacterial cell wall. In the organisms, lysozyme can act as a nonspecific immune molecule against bacterial infections, and as a digestive enzyme in enteron and some mollusks which live on bacteria. Further, lysozyme has the function of inhibiting tumor growth. In 1955, Caselli and Shumacher (Boll Ocul 34:513-533, 1955) reported on the lysozyme-mediated 70% inhibition of neoplastic transformation in cornea of chicken infected by Rous sarcoma virus. Many other experiments indicated that lysozyme participates in the process of tumor diffusion and interacts with phospho- and glucolipid molecule of tumor cells. The inhibition effect on human tumor of lysozyme was reported and patented (1980 Jpn Kokai, Tokkyo Koho 33,409; 1980 Jpn Kokai Tokkyo Koho 33,408). As to the mechanism of lysozyme inhibition on tumor, there are two possibilities: (1) lysozyme directly activates the organism's immunity functions; (2) lysozyme indirectly enhances the organism's immune ability (1989 Anticancer Research 9, 583-592).

The invention is further illustrated by the following examples. It is appreciated that these examples are only intended to illustrate the invention, but not to limit the scope of the invention. For the experimental methods in the following examples, they are performed under routine conditions, e.g., those described by Sambrook. et al., in Molecule Clone: A Laboratory Manual, New York: Cold Spring Harbor Laboratory Press, 1989, or as instructed by the manufacturers, unless otherwise specified.

Example 1

The cloning and sequencing of LYC3 cDNA sequence

1. Amplification with primers

The template was human brain λ gt 11 cDNA library (commercially available from Clontech). PCR with forward primer A1 : 5'-AGAGTGGTGGTGGCTCCACTCTG-3' (SEQ ID NO: 1) and reverse primer B :5'-TGCTGTGCATGGTTCCGTCCATC-3' (SEQ ID NO: 2) was carried out. The PCR condition

for A1/B was 4 mins at 93°C; followed by 35 cycles with 1 min at 93°C, 1 min at 69°C, and 1 min at 72°C; and, finally 5 mins at 72°C. The PCR fragments were detected by electrophoresis. The target fragment was 544bp.

2. Sequencing PCR products

The obtained PCR products were linked with pGEM-T ® vector (Promega) and transformed into E. coli JM103. The plasmids were extracted using QIAprep Plasmid Kit (QIAGEN). The oriented serial deletion of the inserted fragments was carried out with Double-Stranded Nested Deletion Kit (Pharmacia), and the deletants were quickly identified by PCR and arranged in order. The deletants successively cut-off were sequenced with SequiTherm EXCEL™ DNA Sequencing Kit (Epicentre Technologies). A full length cDNA sequence of 544bp was obtained by overlapping the sequences with computer software. The detailed sequence is shown in SEQ ID NO: 3 with an open reading frame (ORF) located at nucleotides 81-521.

According to the resultant full-length cDNA sequence, the amino acid sequence of LYC3 was deduced, having 146 amino acid residues totally. See SEQ ID NO: 4 for its amino acid sequence in details.

Example 2

Homologous comparison

The full length cDNA sequence of LYC3 and the coded protein were used for homologous screening Non-redundant GenBank + EMBL + DDBJ + PDB and GenBank CDS translations + PDB + SwissProt + Spupdate + PIR databases by BLAST algorithm. The result showed that they shared high homology to other members of the lysozyme family. The amino acid sequence of LYC3 shares 51.4% identity and 64.4% similarity with *Trachypithecus francoisi* lysozyme C (gi|1790947) , and 46.6% identity and 59.2% similarity with lysozyme C of ring-necked pheasant (sp|p00702), when analyzed by PCGENE software.

In particular, in amino acid sequence of LYC3, there exists a 19 amino acids signature sequence of lysozyme and alpha-lactoalbumin: **CX_3CX_2(L/M/F)X_3(D/E/N)(L/I)X_5C** [Note: In the sequence, X represents any amino acid, digits such as "2" denote the number of amino acid, "(L/M/H)" represents any of these three amino acids]. Lysozyme and alpha-lactoalbumin are two proteins related closely in evolution (Eur. J. Biochem. 182: 111-118). In the protein of the present invention, the sequence matching the signature is: CRMYCSDLLNPNLKDTVIC (residues 93-111 in SEQ ID NO: 4). It indicates that the LYC3 of the present invention belongs to lysozyme family, and has the relative functions of the lysozyme family.

Lysozyme can lyse cells by hydrolyze the beta(1-4) glycosidic bond between N-acetylmuramic acid (NAM) and N-acetylgluconic acid (NAG) of the bacterial cell wall. In the organisms, lysozyme can act as a nonspecific immune molecule against bacterial infections, and as a digestive enzyme in enteron and some mollusks which live on bacteria.

Lysozyme has important applications in both industry and medicine.

First, in industry (mainly in food industry), lysozyme can be used as a preservative or additive for food. In this respect, the Japanese have developed many use of lysozyme and owe many patents. For example, they use lysozyme as a preservative for fresh fruit, vegetable, soybean milk, marine foods and meat.

Lysozyme can also be used as an additive for infant's foods to simulate human milk (1988, Crit Rev Food Sci Nutr 26(4):359-395).

In respect of pharmaceutical use, lysozyme can be used to cure viral and bacterial infections. For example, EDTA-tris-lysozyme solutions are effective on the pseudomonas cystitis induced by E.coli infection. Lysozyme concentration in human and animal serum is an indicator of infection. Zajaczkowska-Bialowas and Murai studied the relationship between lysozyme activity in saliva and diseases of oral cavity. The result showed that lysozyme had obvious alleviation effect on the symptom of chronic periodontitis. Besides, they found the synergistic effects of lysozyme and some antibiotics. When lysozyme was used alone, even in a large amount, the bacteriolysis effect on *S. aureus* was little. But with the presence of amoxicillin, the lysis effect was enhanced and in proportion to the amount of lysozyme (1988 Crit Rev Food Sci Nutr 26(4):359-395).

Further, lysozyme has the function of inhibiting tumor growth. In 1955, Caselli and Shumacher (1955, Boll Ocul 34:513-533) reported on the lysozyme-mediated 70% inhibition of neoplastic transformation in cornea of chicken infected by Rous sarcoma virus. Many other experiments indicated that lysozyme had some relationship to the inhibition of tumor diffusion (1988 Clin. Expl. Metastasis 6:245-253; 1998 Folia Onclo 10, Suppl A: 219-224; 1988 Eur. J. Cancer Clin. Onco. 124:1737-1743). It is also found that lysozyme interacts with phospho- and glucolipid molecule of tumor cells. The lysozyme's inhibition effect on human tumor was reported. Laterza successfully cured a case of small intestine reticulation sarcoma with diffusion after operation and radiotherapy ("Atti del II Simposium Internazionale sul Lisozima ", Milano. 7-8-9 1961. Vol I, sez V, pp 49-50). Battaglia et al. found that, though lysozyme could not reduce the volume of tumor, it had distinct effects of pain-killing and helping recovery in curing carcinomas of stomach, prostate, uterus and mammary gland ("Atti del II Simposium Internaionale sul Lisozima di Fleming", Milano. 3-4-5 1964. Vol I, sez IV, pp 69-76). In Japan, the application of lysozyme in curing cancer was patented (1980 Jpn Kokai Tokkyo Koho 33, 409; 1980 Jpn Kokai Tokkyo Koho 33,408). Besides, A. Vacca et al. in 1985 reported an attempt of curing multiple myeloma by chemoimmunology with oral lysozyme as an immunomodulating agent. Their experiments indicated that 50% of the patients treated with a large amount of lysozyme had improved immune ability as compared with the controls (Chemiother IV n.2:147-155,1985). As to the mechanism of lysozyme inhibition on tumor, there are two possibilities: (1) lysozyme directly activates the organism's immunity functions; (2) lysozyme indirectly enhances the organism's immune ability (1989 Anticancer Research 9, 583-592).

Example 3

Expression of LYC3 in E. coli

The cDNA sequence encoding LYC3 was amplified with oligonucleotide PCR primers corresponding to 5'- and 3'-end of said DNA sequence, using human brain λ gt 11 cDNA library (Clontech) as a template. The resultant product was used as an insertion fragment.

The sequence of 5'-end oligonucleotide primer was:

5'-TCTCGGATCCATGTTGTTGGCCCTGGTCT-3' (SEQ ID NO: 5).

This primer contained a cleavage site of restriction endonuclease BamH I, followed by 19 nucleotides

of LYC3 coding sequence starting from the start codon.

The sequence of 3'-end primer was:

5'-CCTTGTCGACCTAGAAGTCACAGCCATCC-3'(SEQ ID NO: 6).

This primer contains a cleavage site of restriction endonuclease SalI, a translation terminator and partial LYC3 coding sequence.

These cleavage sites of restriction endonuclease in primers corresponded to the cleavage sites in bacterial expression vector pQE-9 (Qiagen Inc., Chatsworth, CA). Vector pQE-9 encodes an antibiotic resistance (Amp^r), a bacterial replication origin (ori), an IPTG-adjustable promotor/operon (P/O), a ribosome-binding site (RBS), a six-hisitine tag (6-His) and cloning sites of restriction endonuclease.

Vector pQE-9 and insertion fragments were digested by BamHI and SalI, and then linked together, ensuring that the open reading frame started from the bacterial RBS. Then, the linkage mixture was used to transform E.coli M15/rep4 (Qiagen) containing multi-copy of plasmid pREP4 which expressed repressor of lacI and was resistant to kanamycin (Kan^r). Transformants were screened out in LB medium containing Amp and Kan. The positive clones of transformant were cultured overnight in LB liquid medium supplemented with Amp (100ug/ml) and Kan (25ug/ml). The plasmids were extracted. The size and direction of the inserted fragments were verified by HindIII digestion. The sequencing confirmed that LYC3 cDNA fragment was correctly inserted into the vector.

The overnight culture was 1:100-1:250 diluted, inoculated into large volume medium, and cultured until the 600nm optical density (OD_{600}) reached 0.4-0.6. IPTG (isopropylthio-beta-D-galactoside) was added to final concentration of 1mM. By deactivating repressor of LacI, IPTG induced and promoted P/O, thereby increasing the expression of gene. The cells were cultured for another 3-4 hours, and then centrifuged (6000Xg, 20 mins). The inclusions were sonicated, and cell was collected and precipitates was solved in 6M guanidine hydrochloride. After clarification, the dissolved LYC3 in solution were purified by nickel-chelated column chromatography under the conditions suitable for the tight binding of 6-His tagged protein and column. LYC3 was eluted with 6M-guanidine hydrochloride (pH 5.0). The denaturalized proteins in guanidine hydrochloride were precipitated by several methods. First, guanidine hydrochloride was separated by dialysis. Alternatively, the purified protein, which was isolated from nickel-chelated column, bound to the second column with decreased linear gradient of guanidine hydrochloride. The proteins were denatured when binding to the column, and then eluted with guanidine hydrochloride (pH 5.0). Finally, the soluble proteins were dialyzed with PBS, then preserved in glycerol stock solution with the final glycerol concentration of 10% (w/v).

The molecular weight of the expressed protein was 16 kDa, as identified by 12% SDS-PAGE.

Moreover, the sequencing results of the 10 amino acids at the N- and C-terminal of the expressed protein indicated that they were identical to those in SEQ ID NO: 4.

Example 4

Expression of LYC3 in eukaryotic cells (CHO cell line)

In this example, the cDNA sequence encoding LYC3 was amplified with oligonucleotide PCR primers corresponding to 5'- and 3'-end of said DNA sequence, using human brain λ gt 11 cDNA library

(Clontech) as a template. The resultant product was used as an insertion fragment.

The sequence of 5'-end oligonucleotide primer was:

5'-TCTCAAGCTTATGTTGTTGGCCCTGGTCT-3'(SEQ ID NO: 7),

This primer contained a cleavage site of restriction endonuclease HindIII, followed by 20 nucleotides of LYC3 coding sequence starting from the start codon.

The sequence of 3'-end primer was:

5'-CCTTGGATCCCTAGAAGTCACAGCCATCC-3' (SEQ ID NO: 8)

The primer contained a cleavage site of restriction endonuclease BamHI, a translation stop codon, and partial LYC3 coding sequence.

These cleavage sites of restriction endonuclease in primers corresponded to the cleavage sites in expression vector pcDNA3 for CHO cell. This vector encoded two kinds of antibiotic resistance (Amp^r and Neo^r), a phage replication origin (fl ori), a virus replication origin (SV40 ori), a T7 promoter, a virus promoter (P-CMV), a Sp6 promoter, a polyadenylation signal of SV40 and the corresponding polyA sequence thereof, a polyadenylation signal of BGH and the poly A sequence thereof.

The vector pcDNA3 and insertion fragment were digested with HindIII and BamHI, and linked together. Subsequently, E.coli strand DH5 α was transformed with linkage mixture. Transformants were screened out in LB medium containing Amp. The clones containing the needed constructs were cultured overnight in LB liquid medium supplemented with Amp (100 ug/ml). Plasmids were extracted. The sequencing indicated that LYC3 cDNA fragment was correctly inserted into the vector.

Plasmids were transfected into CHO cells by lipofection with Lipofectin Kit (GIBco Life). After transfecting the cells for 48 hours and screening the cells with G418 for 2-3 weeks, the cells and cell supernatant were collected and the enzyme activity of the expressed protein was measured. G418 was removed and the transformants were subcultured continuously. The mixed clonal cells were limiting diluted and the subclones with higher protein activity were selected. The positive subclones were mass cultured by routine methods. 48 hours later, the cells and supernatant were collected. The cells were ultrasonicated. Using 50mM Tris-HCl (pH7.6) solution containing 0.05% Triton as an equilibrium solution and eluent, the active peek of the protein was collected with a pre-balanced Superdex G-75 column. Then, using 50mM Tris-HCl (pH8.0) solution containing 0-1 M NaCl as an eluent, the protein was gradiently washed on a DEAE-Sepharose column balanced with 50mM Tris-HCl (pH8.0) solution. The active peek of the protein was collected. The solution of the expressed protein was dialyzed with PBS (pH7.4), and finally lyophilized and preserved.

The molecular weight of the expressed protein was 16 kDa as identified by 12% SDS-PAGE.

Moreover, the sequencing results of the 10 amino acids at the N- and C-terminal of the expressed protein indicated that they were identical to those in SEQ ID NO: 4.

Example 5

Antibody preparation

Antibodies were produced by immunizing animals with the recombinant proteins obtained in the above examples. The method was as follows: the recombinant proteins were isolated by chromatography, and

stored for use. Alternatively, the protein was isolated by SDS-PAGE electrophoresis, and obtained by cutting eletrophoretic bands from gel. The protein was emulsified with Freund's complete adjuvant of the same volume. The emulsified protein was injected intraperitoneally into mice at a dosage of 50-100ug/0.2ml. 14 days later, the same antigen was emulsified with Freund's incomplete adjuvant and injected intraperitoneally into mice at a dosage of 50-100ug/0.2ml for booster immunization. Booster immunization was carried out every 14 days, for at least three times. The specific activity of the obtained antiserum was evaluated by its ability of precipitating the translation product of LYC3 gene in vitro.

All the documents cited herein are incorporated into the invention as reference, as if each of them is individually incorporated. Further, it is appreciated that, in the above teaching of the invention, the skilled in the art can make certain changes or modifications to the invention, and these equivalents are still within the scope of the invention defined by the appended claims of the present application.

SEQUENCE LISTING

(2) INFORMATION FOR SEQ ID NO: 1:
(i)SEQUENCE CHARACTERISTICS:
(A)LENGTH: 23bp
(B)TYPE: nucleic acid
(C)STRANDEDNESS: single
(D)TOPOLOGY: linear
(ii)MOLECULAR TYPE: oligonucleotide
(xi)SEQUENCE DESCRIPTION: SEQ ID NO: 1:
AGAGTGGTGG TGGCTCCACT CTG 23

(2) INFORMATION FOR SEQ ID NO: 2:
(i)SEQUENCE CHARACTERISTICS:
(A)LENGTH: 23bp
(B)TYPE: nucleic acid
(C)STRANDEDNESS: single
(D)TOPOLOGY: linear
(ii)MOLECULAR TYPE: oligonucleotide
(xi)SEQUENCE DESCRIPTION: SEQ ID NO: 2:
TGCTGTGCAT GGTTCCGTCC ATC 23

(2) INFORMATION FOR SEQ ID NO: 3:
(i)SEQUENCE CHARACTERISTICS:
(A)LENGTH: 544bp
(B)TYPE: nucleic acid
(C)STRANDEDNESS: double
(D)TOPOLOGY: linear
(ii)MOLECULAR TYPE: cDNA
(xi)SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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  1 AGAGTGGTGG TGGCTCCACT CTGCCGCCGC ATAGAAGCCA GGAGCAGGGC TCTCAGAAGG
 61 CGGTGGTGCC AGCTGGGATC ATGTTGTTGG CCCTGGTCTG TCTGCTCAGC TGCCTGCTAC
121 CCTCCAGTGA GGCCAAGCTC TACGGTCGTT GTGAACTGGC CAGAGTGCTA CATGACTTCG
181 GGCTGGACGG ATACCGGGGA TACAGCCTGG CTGACTGGGT CTGCCTTGCT TATTTCACAA
241 GCGGTTTCAA CGCAGCTGCT TTGGACTACG AGGCTGATGG GAGCACCAAC AACGGGATCT
301 TCCAGATCAA CAGCCGGAGG TGGTGCAGCA ACCTCACCCC GAACGTCCCC AACGTGTGCC
361 GGATGTACTG CTCAGATTTG TTGAATCCTA ATCTCAAGGA TACCGTTATC TGTGCCATGA
421 AGATAACCCA AGAGCCTCAG GGTCTGGGTT ACTGGGAGGC CTGGAGGCAT CACTGCCAGG
481 GAAAAGACCT CACTGAATGG GTGGATGGCT GTGACTTCTA GGATGGACGG AACCATGCAC
541 AGCA
```

(2) INFORMATION FOR SEQ ID NO: 4:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 146 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: lineal
(ii) MOLECULE TYPE: polypeptide
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

```
  1 Met Leu Leu Ala Leu Val Cys Leu Leu Ser Cys Leu Leu Pro Ser
 16 Ser Glu Ala Lys Leu Tyr Gly Arg Cys Glu Leu Ala Arg Val Leu
 31 His Asp Phe Gly Leu Asp Gly Tyr Arg Gly Tyr Ser Leu Ala Asp
 46 Trp Val Cys Leu Ala Tyr Phe Thr Ser Gly Phe Asn Ala Ala Ala
 61 Leu Asp Tyr Glu Ala Asp Gly Ser Thr Asn Asn Gly Ile Phe Gln
 76 Ile Asn Ser Arg Arg Trp Cys Ser Asn Leu Thr Pro Asn Val Pro
```

91 Asn Val Cys Arg Met Tyr Cys Ser Asp Leu Leu Asn Pro Asn Leu
106 Lys Asp Thr Val Ile Cys Ala Met Lys Ile Thr Gln Glu Pro Gln
121 Gly Leu Gly Tyr Trp Glu Ala Trp Arg His His Cys Gln Gly Lys
136 Asp Leu Thr Glu Trp Val Asp Gly Cys Asp Phe

(2) INFORMATION FOR SEQ ID NO: 5:
(i)SEQUENCE CHARACTERISTICS:
(A)LENGTH: 29bp
(B)TYPE: nucleic acid
(C)STRANDEDNESS: single
(D)TOPOLOGY: linear
(ii)MOLECULAR TYPE: oligonucleotide
(xi)SEQUENCE DESCRIPTION: SEQ ID NO: 5:
TCTCGGATCC ATGTTGTTGG CCCTGGTCT 29

(2) INFORMATION FOR SEQ ID NO: 6:
(i)SEQUENCE CHARACTERISTICS:
(A)LENGTH: 29bp
(B)TYPE: nucleic acid
(C)STRANDEDNESS: single
(D)TOPOLOGY: linear
(ii)MOLECULAR TYPE: oligonucleotide
(xi)SEQUENCE DESCRIPTION: SEQ ID NO: 6:
CCTTGTCGAC CTAGAAGTCA CAGCCATCC 29

(2) INFORMATION FOR SEQ ID NO: 7:
(i)SEQUENCE CHARACTERISTICS:
(A)LENGTH: 29bp
(B)TYPE: nucleic acid
(C)STRANDEDNESS: single
(D)TOPOLOGY: linear
(ii)MOLECULAR TYPE: oligonucleotide
(xi)SEQUENCE DESCRIPTION: SEQ ID NO: 7:
TCTCAAGCTT ATGTTGTTGG CCCTGGTCT 29

(2) INFORMATION FOR SEQ ID NO: 8:
(i)SEQUENCE CHARACTERISTICS:
(A)LENGTH: 29bp
(B)TYPE: nucleic acid
(C)STRANDEDNESS: single
(D)TOPOLOGY: linear
(ii)MOLECULAR TYPE: oligonucleotide
(xi)SEQUENCE DESCRIPTION: SEQ ID NO: 8:
CCTTGGATCC CTAGAAGTCA CAGCCATCC 29

CLAIMS

1. An isolated DNA molecule comprising a nucleotide sequence encoding a polypeptide having human LYC3 protein activity, wherein said nucleotide sequence shares at least 70% homology to the nucleotide sequence of nucleotides 81-521 in SEQ ID NO: 3, or said nucleotide sequence can hybridize to the nucleotide sequence of nucleotides 81-521 in SEQ ID NO: 3 under moderate stringency.

2. The DNA molecule of Claim 1 wherein said nucleotide sequence encodes a polypeptide comprising the amino acid sequence of SEQ ID NO: 4 .

3. The DNA molecule of Claim 1 wherein said nucleotide sequence comprises the nucleotide sequence of nucleotides 81-521 in SEQ ID NO: 3.

4. An isolated LYC3 polypeptide comprising a polypeptide having the amino acid sequence of SEQ ID NO: 4 , its active fragments, and its active derivatives.

5. The polypeptide of Claim 4 wherein said polypeptide is a polypeptide having the amino acid sequence of SEQ ID NO: 4 .

6. A vector containing the DNA sequence of Claim 1.

7. A host cell transformed by the vector of Claim 6.

8. The host cell of claim 7 wherein it comprises E.coli.

9. The host cell of claim 7 wherein it comprises eukaryotic cell.

10. A method for producing a method for producing a polypeptide having the activity of LYC3 protein, which comprises the steps of :

(a) forming an expression vector of LYC3 protein comprising the nucleotide sequence encoding the polypeptide having the activity of LYC3 protein, wherein said nucleotide sequence is operably linked with an expression regulatory sequences, and said nucleotide sequence shares at least 70% homology to the nucleotide sequence of positions 81-521 in SEQ ID NO: 3;

(b) introducing the vector of step (a) into a host cell, thereby forming a recombinant cell of LYC3 protein;

(c) culturing the recombinant cell of step (b) under the conditions suitable for expression of LYC3 polypeptides;

(d) isolating the polypeptides having the activity of LYC3 protein.

11. The method of Claim 10 wherein said nucleotide sequence comprises nucleotides 81-521 of SEQ ID NO: 3.

12. An antibody specifically bound with the LYC3 polypeptide of Claim 4.

13. A nucleotide molecule wherein it is the antisense sequence of the DNA molecule of Claim 1.

14. A probe wherein it comprises about 8-100 consecutive nucleotides of the DNA molecule of Claim 1.

ABSTRACT

The invention relates to a novel member LYC3 of lysozyme gene family. The invention provides the cDNA sequence encoding for the novel lysozyme, the polypeptide encoded by the sequence, as well as the method for producing said novel human lysozyme utilizing recombinant technology. The invention also provides the use of the novel human lysozyme.

PCT/CN 99/00132

4 09/786024

证 明

本证明之附件是向本局提交的下列专利申请副本

申 请 日: 98 08 31

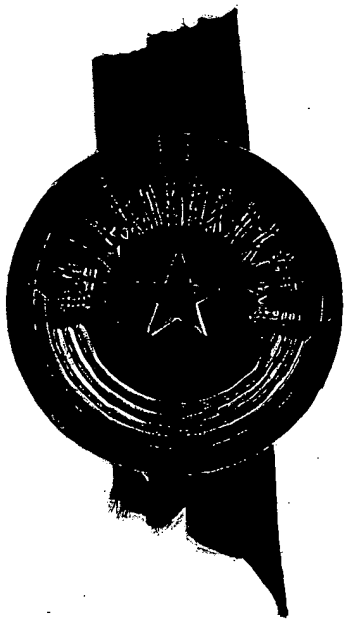
申 请 号: 98 1 11041.X

申 请 类 别: 发 明

发明创造名称: 一种新的人溶菌酶基因、其编码的多肽及制备方法

发明人或设计人: 余 龙 张宏来 傅 强 赵 勇 毕安定

申 请 人: 复旦大学



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中华人民共和国
国家知识产权局局长 姜颖

99 年 09 月 17 日

权 利 要 求 书

1. 一种分离出的DNA分子，其特征在于，它包括：编码具有人LYC3蛋白活性的多肽的核苷酸序列，

所述的核苷酸序列与SEQ ID NO. 3中从核苷酸81-521位的核苷酸序列有至少70%的同源性；或者

所述的核苷酸序列能在中度严紧条件下与SEQ ID NO. 3中从核苷酸81-521位的核苷酸序列杂交.

2.如权利要求1所述的DNA分子，其特征在于，所述的序列编码一多肽，该多肽具有SEQ ID NO. 4所示的序列.

3.如权利要求1所述的DNA分子，其特征在于，该序列具有SEQ ID NO. 3中从核苷酸81-521位的核苷酸序列.

4.一种分离的LYC3蛋白多肽，其特征在于，它包括：具有SEQ ID NO. 4氨基酸序列的多肽、或其活性片段，或其活性衍生物.

5.如权利要求4所述的多肽，其特征在于，该多肽是具有SEQ ID NO. 4序列的多肽.

6.一种载体，其特征在于，它含有权利要求1所述的DNA.

7.一种用权利要求6所述载体转化的宿主细胞.

8.如权利要求7所述的宿主细胞，其特征在于，该细胞是大肠杆菌.

9.如权利要求7所述的宿主细胞，其特征在于，该细胞是真核细胞.

10.一种产生具有LYC3蛋白活性的多肽的方法，其特征在于，该方法包括：

(a)将编码具有LYC3蛋白活性的多肽的核苷酸序列可操作地连于表达调控序列，形成LYC3蛋白表达载体，所述的核苷酸序列与SEQ ID NO. 3中从核苷酸81-521位的核苷酸序列有至少70%的同源性；

(b)将步骤(a)中的表达载体转入宿主细胞，形成LYC3蛋白的重组细胞；

(c)在适合表达LYC3蛋白多肽的条件下，培养步骤(b)中的重组细胞；

(d)分离出具有LYC3蛋白活性的多肽.

11.如权利要求10所述的方法，其特征在于，该序列为SEQ ID NO. 3中从核苷酸81-521位.

12.一种能与权利要求4所述的LYC3蛋白多肽特异性结合的抗体.

13.一种核苷酸分子，其特征在于，它是权利要求1所述DNA分子的反义序列.

14.一种探针分子，其特征在于，它含有权利要求1所述的DNA分子中约8-100个连续核苷酸。

说　明　书

一种新的人溶菌酶基因、其编码的多肽及制备方法

本发明涉及一种新的多核苷酸，由该多核苷酸编码的多肽，这些多核苷酸和多肽的应用，以及所述多核苷酸和所述多肽的生产方法．更具体地说，本发明的多肽被推断鉴定为溶菌酶家族的一个新成员．

溶菌酶广泛存在于生物体的各个部分中，包括各种组织、器官和血清，鸡蛋清中的含量尤为丰富，它主要由特定的腺体上皮细胞或是某些白细胞分泌产生．

溶菌酶最早见诸报导是在1922年由Fleming等发表的论文．自那以后，溶菌酶被从各个不同的角度进行了广泛的研究，例如它晶体结构、蛋白催化结构域、催化动力学、免疫学、分子进化学等方面已发表了大量的文献．溶菌酶已成为研究最广泛、最深入的蛋白质之一．但是，关于溶菌酶基因的研究还开展的很不够．迄今为止只有少数物种的溶菌酶基因已被克隆：如大肠杆菌T4溶菌酶、沙门氏菌P22噬菌体溶菌酶、杆菌φ噬菌体溶菌酶、鸡溶菌酶等等(1983 J. Mol. Biol. 165, 229-248; 1985 Virology 143,280-289.; 1987 Proc. Natl. Acad. Sci. USA, 84, 955-958.; Proc. Natl. Acad. Sci. USA, 77, 5759-5763)，人中有关溶菌酶基因克隆方面的报道也已有相关文献公布(1988 Gene 66, 223-234.).

溶菌酶的主要作用是通过裂解细菌细胞壁的NAM(N-乙酰胞壁酸)和NAG(N-乙酰葡萄糖酸)间的β(1-4)糖苷键而达到裂解细胞的效果．生物体中溶菌酶作为一种非特异性的免疫分子具有抗细菌感染作用，在肠道内及一些依靠细菌生存的软体动物中还被作为一种消化酶起作用．溶菌酶还有抑制肿瘤生长的功能．正是由于溶菌酶具有上述功能，所以它无论在工业上还是在医学上都有重要的应用价值．

本发明的一个目的是提供一种新的多核苷酸，该多核苷酸编码溶菌酶基因家族的一个新成员，本发明的溶菌酶被命名为LYC3.

本发明的另一个目的是提供一种新的溶菌酶蛋白家族成员，该酶被命名为LYC3.

本发明的再一个目的是提供一种利用重组技术生产所述的新的人溶菌酶的方法.

本发明还涉及这种人溶菌酶基因序列和多肽的应用.

在本发明的一个方面，提供了一种分离出的DNA分子，它包括：编码具有人LYC3蛋白活性的多肽的核苷酸序列，所述的核苷酸序列与SEQ ID NO. 3中从核苷酸81-521位的核苷酸序列有至少70%的同源性；或者所述的核苷酸序列能在中度严紧条件下与SEQ ID NO. 3中从核苷酸81-521位的核苷酸序列杂交．较佳地，所述的序列编码一多肽，该多肽具有SEQ ID NO. 4所示的序列．更佳地，该序列是SEQ ID NO. 4．

在本发明的另一方面，提供了一种分离的LYC3蛋白多肽，它包括：具有SEQ ID NO. 4氨基酸序列的多肽、或其活性片段，或其活性衍生物．较佳地，该多肽是具有SEQ ID NO. 4序列的多肽．

在本发明的另一方面，提供了一种载体，它含有上述分离出的DNA．

在本发明的另一方面，提供了一种所述载体转化的宿主细胞．

在本发明的另一方面，提供了一种产生具有LYC3蛋白活性的多肽的方法，该方法包括：

(a)将编码具有LYC3蛋白活性的多肽的核苷酸序列可操作地连于表达调控序列，形成LYC3蛋白表达载体，所述的核苷酸序列与SEQ ID NO. 3中从核苷酸81-521位的核苷酸序列有至少70%的同源性；

(b)将步骤(a)中的表达载体转入宿主细胞，形成LYC3蛋白的重组细胞；

(c)在适合表达LYC3蛋白多肽的条件下，培养步骤(b)中的重组细胞；

(d)分离出具有LYC3蛋白活性的多肽．

在本发明的一个具体实施方案中，本发明的分离的多核苷酸全长为583个核苷酸，其详细序列见SEQ ID NO. 3，其中开放读框位于81-521位核苷酸．

在本发明中，“分离的”、“纯化的”或“基本纯的”DNA是指，该DNA或片段已从天然状态下位于其两侧的序列中分离出来，还指该DNA或片段已经与天然状态下伴随核酸的组份分开，而且已经与在细胞中伴随其的蛋白质分开．

在本发明中，术语“LYC3蛋白(或多肽)编码序列”指编码具有LYC3蛋白活性的多肽的核苷酸序列，如SEQ ID NO. 3中81-521位核苷酸序列及其简并序列．该简并序列是指，位于SEQ ID NO. 3序列的编码框81-521位核苷酸中，有一个或多个密码子被编码相同氨基酸的简并密码子所取代后而产生的序列．由于密码子的简并性，所以与SEQ ID NO. 3中81-521位核苷酸序列同源性低至约70%的简并序列也能编码出SEQ ID NO. 4所述的序列．该术语还包括能在中度严紧条件下与SEQ ID NO. 3中从核苷酸81-521位的核苷酸序列杂交的核苷酸序列．此外，该术

语还包括与SEQ ID NO. 3中从核苷酸81-521位的核苷酸序列的同源性至少70%，较佳地至少80%，更佳地至少90%的核苷酸序列.

该术语还包括能编码具有与人LYC3相同功能的蛋白的、SEQ ID NO. 3序列的变异形式. 这些变异形式包括(但并不限于): 若干个(通常为1－90个，较佳地1－60个，更佳地1－20个，最佳地1－10个)核苷酸的缺失、插入和/或取代，以及在5'和/或3'端添加数个(通常为60个以内，较佳地为30个以内，更佳地为10个以内，最佳地为5个以内)核苷酸.

在本发明中，“基本纯的”蛋白质或多肽是指其至少占样品总物质的至少20%，较佳地至少50%，更佳地至少80%，最佳地至少90%(按干重或湿重计). 纯度可以用任何合适的方法进行测量，如用柱层析、PAGE或HPLC法测量多肽的纯度. 基本纯的多肽基本上不含天然状态下的伴随其的组分.

在本发明中，术语“LYC3蛋白多肽”指具有LYC3蛋白活性的SEQ ID NO. 4序列的多肽. 该术语还包括具有与人溶菌酶相同功能的、SEQ ID NO. 4序列的变异形式. 这些变异形式包括(但并不限于): 若干个(通常为1－50个，较佳地1－30个，更佳地1－20个，最佳地1－10个)氨基酸的缺失、插入和/或取代，以及在C末端和/或N末端添加一个或数个(通常为20个以内，较佳地为10个以内，更佳地为5个以内)氨基酸. 例如，在本领域中，用性能相近或相似的氨基酸进行取代时，通常不会改变蛋白质的功能. 又比如，在C末端和/或N末端添加一个或数个氨基酸通常也不会改变蛋白质的功能. 该术语还包括LYC3蛋白的活性片段和活性衍生物.

该多肽的变异形式包括: 同源序列、等位变异体、天然突变体、诱导突变体、在高或低的严谨度条件下能与LYC3 DNA 杂交的DNA所编码的蛋白、以及利用抗LYC3多肽的抗血清获得的多肽或蛋白. 本发明还提供了其他多肽，如包含LYC3多肽或其片段的融合蛋白. 除了几乎全长的多肽外，本发明还提供了LYC3多肽的可溶性片段. 通常，该片段具有LYC3多肽序列的至少约10个连续氨基酸，通常至少约30个连续氨基酸，较佳地至少约50个连续氨基酸，更佳地至少约80个连续氨基酸，最佳地至少约100个连续氨基酸.

发明还提供LYC3蛋白或多肽的类似物. 这些类似物与天然LYC3多肽的差别可以是氨基酸序列上的差异，也可以是不影响序列的修饰形式上的差异，或者兼而有之. 这些多肽包括天然或诱导的遗传变异体. 诱导变异体可以通过各种技术得到，如通过辐射或暴露于诱变剂而产生随机诱变，还可通过定点诱变法或其他已知分子生物学的技术. 类似物还可以包括具有不同于天然L-氨基酸的残基(如D-

氨基酸)的类似物，以及具有非天然存在的或合成的氨基酸(如β、γ-氨基酸)的类似物．应理解，本发明的多肽并不限于上述例举的代表性的多肽．

修饰(通常不改变一级结构)形式包括：体内或体外的多肽的化学衍生形式如乙酰化或羧基化．修饰还包括糖基化，如那些在多肽的合成和加工中或进一步加工步骤中进行糖基化修饰而产生的多肽．这种修饰可以通过将多肽暴露于进行糖基化的酶(如哺乳动物的糖基化酶或去糖基化酶)而完成．修饰形式还包括具有磷酸化氨基酸残基(如磷酸酪氨酸，磷酸丝氨酸，磷酸苏氨酸)的序列．还包括被修饰从而提高了其抗蛋白水解性能或优化了溶解性能的多肽．

本发明还包括LYC3多肽编码序列的反义序列．这种反义序列可用于抑制细胞内LYC3的表达．

本发明还包括一种探针分子，该分子通常具有LYC3多肽编码序列的8-100个，较佳地15-50个连续核苷酸．该探针可用于检测样品中是否存在编码LYC3的核酸分子．

本发明还包括检测LYC3核苷酸序列的方法，它包括用上述的探针与样品进行杂交，然后检测探针是否发生了结合．较佳地，该样品是PCR扩增后的产物，其中PCR扩增引物对应于LYC3多肽的编码序列，可位于该编码序列的两侧或中间．引物长度一般为20-50个核苷酸．

在本发明中，可选用本领域已知的各种载体，如市售的载体．

在本发明中，术语“宿主细胞”包括原核细胞和真核细胞．常用的原核宿主细胞的例子包括大肠杆菌、枯草杆菌等．常用的真核宿主细胞包括酵母细胞，昆虫细胞、和哺乳动物细胞．较佳地，该宿主细胞是真核细胞，如CHO细胞、COS细胞等．

另一方面，本发明还包括对LYC3 DNA或是其片段编码的多肽具有特异性抗体，尤其是单克隆抗体．这里，“特异性”是指抗体能结合于LYC3基因产物或片段．较佳地，指那些能与LYC3基因产物或片段结合但不识别和结合于其它非相关抗原分子的抗体．本发明中抗体包括那些能够结合并抑制LYC3蛋白的分子，也包括那些并不影响LYC3蛋白功能的抗体．本发明还包括那些能与修饰或未经修饰形式的LYC3基因产物结合的抗体．

本发明不仅包括完整的单克隆或多克隆抗体，而且还包括具有免疫活性的抗体片段，如Fab'或$(Fab)_2$片段；抗体重链；抗体轻链；遗传工程改造的单链Fv分子(Ladner等人，美国专利No. 4,946,778)；或嵌合抗体，如具有鼠抗体结合特异性但仍保留来自人的抗体部分的抗体．

本发明的抗体可以通过本领域内技术人员已知的各种技术进行制备，例如，纯化的LYC3基因产物或者其具有抗原性的片段，可被施用于动物以诱导多克隆抗体的产生，与之相似的，表达LYC3或其具有抗原性的片段的细胞可用来免疫动物来生产抗体．本发明的抗体也可以是单克隆抗体．此类单克隆抗体可以利用杂交瘤技术来制备(见 Kohler 等人，Nature 256;495, 1975; Kohler 等人，Eur.J.Immunol. 6:511, 1976; Kohler 等人，Eur.J.Immunol. 6:292, 1976; Hammerling 等人，In Monoclonal Antibodies and T Cell Hybridomas, Elsevier, N.Y., 1981)．本发明的抗体包括能阻断LYC3功能的抗体以及不影响LYC3功能的抗体．本发明的各类抗体可以利用LYC3基因产物的片段或功能区，通过免疫技术获得．这些片段或功能区可以利用重组方法制备或利用多肽合成仪合成．与LYC3基因产物的未修饰形式结合的抗体可以用原核细胞(例如*E. Coli*)中生产的基因产物来免疫动物而产生；与翻译后修饰形式结合的抗体(如糖基化或磷酸化的蛋白或多肽)，可以用真核细胞(例如酵母或昆虫细胞)中产生的基因产物来免疫动物而获得．

在本发明的一个实施方案中，本发明的多核苷酸全长为544个核苷酸，其详细序列见SEQ ID NO.3，其中开放读框位于106-252位核苷酸．该多核苷酸是如此获得的，以人脑λ gt11cDNA文库(购自Clontech公司)为模板，合成正向引物A1: 5'-AGAGTGGTGGTGGCTCCACTCTG -3' 和反向引物B1: 5'-TGCTGTGCATGGTTCCGTCCATC-3'进行PCR，获得544bp的目的片段．测序后得到SEQ ID NO.3的全长cDNA序列．

根据同源比较的结果，本发明的核苷酸序列及其编码的蛋白质序列与不同来源的溶菌酶显示了显著的同源性，因此，这表明它是溶菌酶家族的一个新成员，并且具有溶菌酶家族蛋白的一些重要功能．

溶菌酶可以通过裂解细菌细胞壁的NAM(N-乙酰胞壁酸)和NAG(N-乙酰葡萄糖酸)间的β(1-4)糖苷键而达到裂解细胞的效果．生物体中溶菌酶作为一种非特异性的免疫分子具有抗细菌感染作用，在肠道内及一些依靠细菌生存的软体动物中还被作为一种消化酶起作用．溶菌酶还有抑制肿瘤生长的功能．1955年，Caselli 和 Shumacher(1955 Boll Ocul 34:513-533.)就报导了在由劳氏肉瘤病毒引起的鸡的角膜肿瘤形成中，溶菌酶介导了对这一过程的70%的抑制作用．许多其它的实验也说明溶菌酶参与了肿瘤的扩散及与肿瘤细胞的磷酸基和糖脂类分子的相互作用．溶菌酶对人类肿瘤的抑制作用也已见诸报导并取得专利(1980 Jpn Kokai Tokkyo Koho 33, 409.; 1980 Jpn Kokai Tokkyo Koho 33, 408)．至于溶菌酶对肿瘤抑制的机制，有两种可能性，一是溶菌酶直接活化了生物体的免疫功能，二是它

间接增强了生物的免疫能力(1989 Anticancer Reserch 9,583-592).

下面结合具体实施例，进一步阐述本发明．应理解，这些实施例仅用于说明本发明而不用于限制本发明的范围．下列实施例中未注明具体条件的实验方法，通常按照常规条件如Sambrook等人， 分子克隆: 实验室手册(New York: Cold Spring Harbor Laboratory Press, 1989)中所述的条件，或按照制造厂商所建议的条件．

实施例1

LYC3的cDNA序列的克隆和测定

1．引物扩增

以人脑λgt11cDNA文库(购自Clontech公司)为模板，用一对寡核苷酸为引物——A1: 5'- AGAGTGGTGGTGGCTCCACTCTG -3' (SEQ ID NO.1)为正向引物，寡核苷酸B1: 5'- TGCTGTGCATGGTTCCGTCCATC -3'(SEQ ID NO.2)为反向引物，进行PCR．PCR条件为93℃4分钟，随之以93℃1分钟、69℃1分钟和72℃1分钟进行35个循环，最后72℃延伸5分钟．电泳检测得到的PCR片断为544bp的目的片段．

2．PCR产物的测序

将如上获得的PCR扩增产物与pGEM-T®载体(Promega)连接，转化大肠杆菌JM103，用QIAprep Plasmid试剂盒(QIAGEN)提取质粒，用双链嵌套式缺失试剂盒(Pharmacia)对插入片段进行定向系列缺失，然后用PCR对缺失子进行快速鉴定及排序．用SequiTherm EXCEL™ DNA测序试剂盒(Epicentre Technologies)对依次截短的缺失子进行测序，最后用电脑软件拼接顺序，获得全长cDNA序列，共544bp，详细序列见SEQ ID NO.3，其中开放读框位于81-521位核苷酸．

根据得到的全长cDNA序列推导出LYC3的氨基酸序列，共146个氨基酸残基，其氨基酸序列详见SEQ ID NO.4．

实施例2

同源比较

用LYC3的全长cDNA序列及其编码蛋白在Non-redundant GenBank+EMBL+DDBJ+PDB数据库及Non-redundant GenBank CDS translations+PDB+SwissProt+Spupdate+PIR数据库中用BLAST进行核酸和蛋白同源检索．结果发现它们溶菌酶家族成员显示了较高的同源性，如用PCGENE软件

分析，它与Trachypithecus francoisi的溶菌酶C(gi|1790947)在蛋白水平上显示了51.4%的同一性和64.4%的相似性；又如，它与ring-necked pheasant(注：一种雉)的溶菌酶C(sp|p00702)在蛋白水平上显示了46.6%的同一性和59.2%的相似性.

特别是在LYC3的氨基酸序列中存在由19个氨基酸组成的被认为是溶菌酶和α乳清蛋白的特征性序列——$CX_3CX_2(L/M/F)X_3(D/E/N)(L/I)X_5C$[注：该序列中X为任意氨基酸，“2”等数字为氨基酸数目，“(L/M/H)”表示从这3个氨基酸中任选一个氨基酸](溶菌酶和α乳清蛋白是在进化上密切相关的两类蛋白(Eur.J.Biochem. 182:111-118)). 在本发明的蛋白中符合上述模式的序列片段是：CRMYCSDLLNPNLKDTVIC(SEQ ID NO. 4中93-111位). 这更确定了本发明的LYC3也属于溶菌酶家族，并且具有溶菌酶家族的相关功能.

溶菌酶可以通过裂解细菌细胞壁的NAM(N-乙酰胞壁酸)和NAG(N-乙酰葡萄糖酸)间的β(1-4)糖苷键而达到裂解细胞的效果.生物体中溶菌酶作为一种非特异性的免疫分子具有抗细菌感染作用，在肠道内及一些依靠细菌生存的软体动物中还被作为一种消化酶起作用.

溶菌酶无论在工业上还是在医学上都有重要的用途.

首先在工业上(主要是食品工业)，溶菌酶可以作为食品的保鲜剂或添加剂使用. 在这方面，日本人已做了大量应用并拥有许多专利. 比如他们已将溶菌酶用作新鲜水果蔬菜、豆奶、海洋食品和肉类，葡萄酒等的保鲜剂. 溶菌酶还可以用作婴儿食品的添加剂以使其更接近人乳的成份(1988 Crit Rev Food Sci Nutr 26(4):359-395).

药用方面，溶菌酶可以用来治疗病毒和细菌感染. 如EDTA-tris-溶菌酶溶液对于治疗大肠菌感染引起的假单孢菌属膀胱炎有效. 人和动物血清中溶菌酶的水平可以作为是否受到感染的一种标志. Zajaczkowska-Bialowas、Murai等研究了唾液溶菌酶活性和口腔疾病间的关系. 他们的研究显示，溶菌酶对慢性牙周炎的症状具有明显的缓解作用. 另外，还发现溶菌酶与某些抗生素具有协同作用，例如溶菌酶在单独使用的时候，即便用量较大对S.aureus的溶菌作用也很小，但在阿莫西林存在的情况下其溶菌作用增强并与溶菌酶的量成正比(1988 Crit Rev Food Sci Nutr 26(4):359-395).

溶菌酶还有抑制肿瘤生长的功能. 1955年，Caselli 和Shumacher(1955 Boll Ocul 34:513-533.)就报导了在由劳氏肉瘤病毒引起的鸡的角膜肿瘤形成中，溶菌酶介导了对这一过程的70%的抑制作用. 许多其它的实验也说明溶菌酶与抑制肿瘤的扩散有关(1988 Clin Expl Mctastasis 6:245-253; 1998 Folia Oncol 10,suppl

A:219-224:1988 Eur J Cancer Clin Oncol24:1737-1743). 溶菌酶还被发现能与肿瘤细胞的磷酸基和糖脂类分子的相互作用(1988 Crit Rev Food Sci Nutr 26(4):359-395). 溶菌酶对人类肿瘤的抑制作用也已见诸报导,Laterza就用溶菌酶成功的治疗了一例手术和放疗后发生转移的小肠网状肉瘤病例(《Atti del II Simposium Internazionale sul Lisozima》, Milano. 7-8-9 1961.Vol I, sez V,pp 49-50); Battaglia等在用溶菌酶治疗胃癌、前列腺癌、子宫癌、乳房癌的探索中发现，溶菌酶虽不能使肿瘤体积减小，但具有明显的减轻疼痛和辅助恢复的作用(《Atti del II Simposium Internazionale sul Lisozima di Fleming》, Milano. 3-4-5 1964.Vol I, sez IV,pp 69-76); 在日本，溶菌酶在治疗癌症方面的应用已取得专利(1980 Jpn Kokai Tokkyo Koho 33, 409.; 1980 Jpn Kokai Tokkyo Koho 33, 408); 另外，A. Vacca.等在1985年报道了在化学免疫疗法中使用口服溶菌酶作为免疫调节剂治疗多种骨髓瘤(multiple myeloma)的尝试，他们的实验显示在接受大剂量溶菌酶治疗的患者中50%的人免疫能力较之对照组增强了(Chemiother IV n.2:147-155,1985). 至于溶菌酶对肿瘤抑制的机制，有两种可能性，一是溶菌酶直接活化了生物体的免疫功能，二是它间接增强了生物的免疫能力(1989 Anticancer Reserch 9,583-592).

实施例3

LYC3在大肠杆菌中的表达

编码LYC3的cDNA序列用对应于该DNA序列的5'和3'端的PCR寡核苷酸引物，用人脑λgt11cDNA文库(购自Clontech公司)为模板进行扩增，以合成插入片段.

5'寡核苷酸引物序列为

5'- TCTCGGATCCATGTTGTTGGCCCTGGTCT -3'(SEQ ID NO.5),

该引物含有BamHI限制性内切酶的酶切位点，接之是由起始密码子开始的LYC3编码序列的19个核苷酸;

3' 寡核苷酸引物序列为

5'- CCTTGTCGACCTAGAAGTCACAGCCATCC-3'(SEQ ID NO.6),

该引物含有SalI限制性内切酶的酶切位点，一个翻译终止子和LYC3的编码序列.

限制性内切酶的酶切位点对应于细菌表达载体pQE-9(Qiagen Inc., Chatsworth, CA)上的限制性内切酶酶切位点，该质粒载体编码抗生素抗性(Amp^r)、一个细菌复制起点(ori)、一个IPTG-可调启动子/操纵子(P/O)、一个核糖体结合位点(RBS)、

一个6-组氨酸标记物(6-His)以及限制性内切酶克隆位点.

用BamHI和SalI消化pQE-9载体，随后将插入片段连接到pQE-9载体并保持开放读框在细菌RBS起始．随后用连接混合物转化购自Qiagen，商品名为M15/rep4的E.coli菌株，M15/rep4含有多拷贝的质粒pREP4，其表达lacI阻遏物并携带卡那霉素抗性(Kan^r)．在含有Amp和Kan的LB培养皿上筛选转化子，在补加Amp(100μg/ml)和Kan(25μg/ml)的LB液体培养基中过夜培养(O/N)含所需构建物的阳性转化子克隆．抽提质粒，用HindIII酶切鉴定插入片段大小及方向，测序验证结果表明LYC3的cDNA插入片段已正确装入载体.

过夜(O/N)培养物以1：100-1：250的稀释率稀释，然后接种到大体积培养基中，培养细胞生长至600光密度(OD_{600})为0.4-0.6时，加入IPTG(“异丙基硫代-β-D-半乳糖苷”)至终浓度为1mM．通过使lacI阻遏物失活，IPTG诱导启动P/O导致基因表达水平提高．继续培养细胞3-4小时，随后离心(6000 × g，20分钟)．超声裂解包涵体，收集细胞并将细胞沉淀溶于6M的盐酸胍中．澄清后，通过在能使含6-His标记物蛋白紧密结合的条件下，用镍-螯合柱层析从溶液中纯化溶解的LYC3．用6M盐酸胍(pH5.0)从柱中洗脱LYC3．可用几种方法从盐酸胍中变性沉淀蛋白．首先，使用透析步骤除去盐酸胍，或者从镍-螯合柱中分离出的纯化蛋白可以结合到第二个柱中，该柱中具有递减的线性盐酸胍梯度．在结合到该柱时蛋白质变性，随后用盐酸胍(pH5.0)洗脱．最后，将可溶的蛋白质用PBS进行透析，然后将蛋白质保存在终浓度为10%(w/v)甘油的贮存液中.

用12%的SDS-PAGE胶进行电泳，鉴定表达蛋白的分子量大小为16KDa.

此外，用常规方法对表达蛋白的N端和C端各10个氨基酸长度的氨基酸进行测序，发现与SEQ ID NO.4的序列一致.

实施例4

LYC3在真核细胞(CHO细胞株)中的表达

在该实施例中，将编码LYC3的cDNA序列用对应于该DNA序列的5'和3'端的PCR寡核苷酸引物，用人脑λgt11cDNA文库(购自Clontech公司)为模板进行扩增，以合成插入片段.

PCR反应中使用的5'寡核苷酸引物序列为:

5'-TCTCAAGCTTATGTTGTTGGCCCTGGTCT- 3'(SEQ ID NO.7),

该引物含有HindIII限制性限制性内切酶的酶切位点，接之是由起始密码子开始的LYC3编码序列的19个核苷酸;

3'端引物序列为:

5' -CCTTGGATCCCTAGAAGTCACAGCCATCC- 3' (SEQ ID NO.8)

该引物含有BamHI限制性限制性内切酶的酶切位点、一个翻译终止子和LYC3的编码序列.

引物上的限制性内切酶的酶切位点对应于CHO细胞表达载体pcDNA3上的限制性内切酶酶切位点，该质粒载体编码抗生素抗性(Amp^r和Neo^r)、一个噬菌体复制起点(f1 ori)、一个病毒复制起点(SV40 ori)、一个T7启动子、一个病毒启动子(P-CMV)、一个Sp6启动子、一个SV40启动子、一个SV40加尾信号和相应的polyA顺序、一个BGH加尾信号和相应的polyA顺序.

用HindIII、BamHI消化pcDNA3载体，随后将插入片段连接到pcDNA3载体.随后用连接混合物转化E.coli DH5α菌株．在含有Amp的LB培养皿上筛选转化子，在补加Amp(100μg/ml)的LB液体培养基中过夜培养(O/N)含所需构建物的克隆．抽提质粒，测序验证结果表明LYC3的cDNA插入片段已正确装入载体.

质粒转染是采用脂转染法，用Lipofectin试剂盒(GiBco Life)进行的．转染48小时后，经2-3周的持续G418加压筛选，收集细胞及细胞上清测定表达蛋白酶活力．去G418，连续传代培养；对混合克隆细胞极限稀释，选择具有较高蛋白活性的细胞亚克隆．按常规方法大量培养上述阳性亚克隆．48小时后，开始收集细胞及上清，用超声裂解方法破碎细胞．以含0.05%Triton的50mM Tris · HCl(pH7.6)溶液为平衡液及洗脱液，用经预平衡的Superdex G-75柱收集上述蛋白的活性峰.再用50mM Tris · HCl(pH8.0)平衡的DEAE-Sepharose柱，以含0-1M NaCl的50mM Tris · HCl(pH8.0)溶液为洗脱液进行梯度洗脱，收集上述蛋白的活性峰．然后以PBS(pH7.4)为透析液对表达蛋白溶液进行透析．最后冻干保存.

用12%的SDS-PAGE胶进行电泳，鉴定表达蛋白的分子量大小为16KDa.

此外，用常规方法对表达蛋白的N端和C端各10个氨基酸长度的氨基酸进行测序，发现与SEQ ID NO.4的序列一致.

实施例5

制备抗体

将实施例3或实施例4获得的重组蛋白用来免疫动物以产生抗体，具体如下.重组分子用层析法进行分离后备用．也可用SDS-PAGE凝胶电泳法进行分离，将电泳条带从凝胶中切下，并用等体积的完全Freund's佐剂乳化．用50-100μg/0.2ml乳化过的蛋白，对小鼠进行腹膜内注射．14天后，用非完全Freund's佐剂乳化的

同样抗原对小鼠以50-100μg/0.2ml的剂量进行腹膜内注射以加强免疫，每隔14天进行一次加强免疫，至少进行三次．获得的抗血清的特异反应活性用它在体外沉淀LYC3基因翻译产物的能力加以评估．

在本发明提及的所有文献都在本申请中引用作为参考，就如同每一篇文献被单独引用作为参考那样．此外应理解，在阅读了本发明的上述讲授内容之后，本领域技术人员可以对本发明作各种改动或修改，这些等价形式同样落于本申请所附权利要求书所限定的范围．

序 列 表

(2)SEQ ID NO.1的信息

(i)序列特征

(A)长度: 23碱基

(B)类型: 核酸

(C)链性: 单链

(D)拓扑结构: 线性

(ii)分子类型: 寡核苷酸

(xi)序列描述: SEQ ID NO.1:

AGAGTGGTGG TGGCTCCACT CTG 23

(2)SEQ ID NO.2的信息

(i)序列特征

(A)长度: 23碱基

(B)类型: 核酸

(C)链性: 单链

(D)拓扑结构: 线性

(ii)分子类型: 寡核苷酸

(xi)序列描述: SEQ ID NO : 2

TGCTGTGCAT GGTTCCGTCC ATC 23

(2)SEQ ID NO.3的信息:

(i)序列特征:

(A)长度: 544bp

(B)类型: 核酸

(C)链性: 单链

(D)拓扑结构: 线性

(ii)分子类型: cDNA

(xi)序列描述: SEQ ID NO.3

```
  1 AGAGTGGTGGTGGCTCCACTCTGCCGCCGCATAGAAGCCAGGAGCAGGGCTCTCAGAAGG
 61 CGGTGGTGCCAGCTGGGATCATGTTGTTGGCCCTGGTCTGTCTGCTCAGCTGCCTGCTAC
121 CCTCCAGTGAGGCCAAGCTCTACGGTCGTTGTGAACTGGCCAGAGTGCTACATGACTTCG
181 GGCTGGACGGATACCGGGGATACAGCCTGGCTGACTGGGTCTGCCTTGCTTATTTCACAA
241 GCGGTTTCAACGCAGCTGCTTTGGACTACGAGGCTGATGGGAGCACCAACAACGGGATCT
301 TCCAGATCAACAGCCGGAGGTGGTGCAGCAACCTCACCCCGAACGTCCCCAACGTGTGCC
361 GGATGTACTGCTCAGATTTGTTGAATCCTAATCTCAAGGATACCGTTATCTGTGCCATGA
421 AGATAACCCAAGAGCCTCAGGGTCTGGGTTACTGGGAGGCCTGGAGGCATCACTGCCAGG
481 GAAAAGACCTCACTGAATGGGTGGATGGCTGTGACTTCTAGGATGGACGGAACCATGCAC
541 AGCA
```

(2)SEQ ID NO.4的信息:

(i)序列特征:

(A)长度: 146个氨基酸

(B)类型: 氨基酸

(D)拓扑结构: 线性

(ii)分子类型: 多肽

(xi)序列描述: SEQ ID NO.4

```
  1 Met Leu Leu Ala Leu Val Cys Leu Leu Ser Cys Leu Leu Pro Ser
 16 Ser Glu Ala Lys Leu Tyr Gly Arg Cys Glu Leu Ala Arg Val Leu
 31 His Asp Phe Gly Leu Asp Gly Tyr Arg Gly Tyr Ser Leu Ala Asp
 46 Trp Val Cys Leu Ala Tyr Phe Thr Ser Gly Phe Asn Ala Ala Ala
 61 Leu Asp Tyr Glu Ala Asp Gly Ser Thr Asn Asn Gly Ile Phe Gln
 76 Ile Asn Ser Arg Arg Trp Cys Ser Asn Leu Thr Pro Asn Val Pro
 91 Asn Val Cys Arg Met Tyr Cys Ser Asp Leu Leu Asn Pro Asn Leu
106 Lys Asp Thr Val Ile Cys Ala Met Lys Ile Thr Gln Glu Pro Gln
121 Gly Leu Gly Tyr Trp Glu Ala Trp Arg His His Cys Gln Gly Lys
136 Asp Leu Thr Glu Trp Val Asp Gly Cys Asp Phe
```

(2)SEQ ID NO.5的信息

(i)序列特征

(A)长度: 29碱基

(B)类型: 核酸

(C)链性: 单链

(D)拓扑结构: 线性

(ii)分子类型: 寡核苷酸

(xi)序列描述: SEQ ID NO.5:

TCTCGGATCC ATGTTGTTGG CCCTGGTCT29

(2)SEQ ID NO.6的信息

(i)序列特征

(A)长度: 29碱基

(B)类型: 核酸

(C)链性: 单链

(D)拓扑结构: 线性

(ii)分子类型: 寡核苷酸

(xi)序列描述: SEQ ID NO.6:

CCTTGTCGAC CTAGAAGTCA CAGCCATCC29

(2)SEQ ID NO.7的信息

(i)序列特征

(A)长度: 29碱基

(B)类型: 核酸

(C)链性: 单链

(D)拓扑结构: 线性

(ii)分子类型: 寡核苷酸

(xi)序列描述: SEQ ID NO.7:

TCTCAAGCTT ATGTTGTTGG CCCTGGTCT29

(2)SEQ ID NO.8的信息

(i)序列特征

(A)长度: 29碱基

(B)类型: 核酸

(C)链性: 单链

(D)拓扑结构: 线性

(ii)分子类型: 寡核苷酸

(xi)序列描述: SEQ ID NO.8:

CCTTGGATCC CTAGAAGTCA CAGCCATCC 29